



Assessment of Dermal Irritation and Acute Toxicity Potential of Extracts from *Synadenium glaucescens* on Healthy Rabbits, Wistar Albino Rats and Albino Mice

Vitus A. Nyigo^{1,2*}, Robinson Mdegela¹, Faith Mabiki³ and Hamisi M. Malebo²

¹Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, P.O.Box 3021 Morogoro, Tanzania.

²Department of Traditional Medicine Research, National Institute for Medical Research, 3 Barack Obama Drive, P.O.Box 9653, 11101 Dar es Salaam, Tanzania.

³Department of Physical Sciences, Sokoine University of Agriculture, P.O.Box 3038, Morogoro, Tanzania.

Authors' contributions

This work was carried out in collaboration with all authors, they were all responsible in the design of study. Author VAN wrote the protocol and was responsible for data collection analysis and the first drafting of the manuscript. All authors contributed to critical review of the protocol and manuscript. They also read and approved the final manuscript. The corresponding author had the final responsibility to submit the manuscript for publication.

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ABSTRACT

Aim: This study was conceived for the purpose of evaluating dermal toxicity potentials of extracts from *S. glaucescens* which is known for many traditional application in human and animals including healing wounds, boils, HIV, worms and application on the swollen lymph nodes of cattle suffering from east coast fever (ECF). This followed the scanty availability of information regarding

*Corresponding author: E-mail: nyigoo@yahoo.com;

dermal toxicity of this and many other plants in Tanzania despite the growing practice of utilizing plant products and extracts respectively to control and treat ectoparasites, and skin disorders.

Materials and Methods: The dried plant materials were subjected to sequential solvent extraction using organic and aqueous solvents. All test animals were obtained from Sokoine University of Agriculture (SUA), Tanzania. Thus, irritation, and acute dermal toxicity tests were respectively conducted in healthy rabbits and albino rats using the Organization for Economic Cooperation and Development (OECD) guidelines. Albino mice were used to test skin sensitization using method developed by Sailstad.

Results and Discussion: Irritation indices ranged from 3.2 and 0.05, thus according to Draize these are considered as mild and moderate irritants since none of them could reach PII of 5. On the other hand, findings from acute dermal toxicity tests showed no any overt signs of toxicity after two weeks of treatment. Similarly the extracts did not produce any sensitization reaction based on the mouse ear diameter taken by vernier calipers.

Conclusion: Findings from this study have shown that, extracts from dried plant of *S. glaucescens* exhibit neither sensitization nor acute dermal toxicity effects except for mild to moderate irritancy. The findings therefore suggests that extracts from dried plant parts of *S. glaucescens* under the short term use of different extracts from dried leaves and root barks applied on skin of animals do not cause any adverse effects both externally and internally.

Keywords: Irritation; sensitization; acute; pathology.

1. INTRODUCTION

Treatment of various ailments through traditional medicines is a common practice in many countries around the world [1,2]. Skin disorders are among diseases that are managed by traditional medicines [3-5]. These disorders occur worldwide and they affect people of all ages from neonates to the elderly [1]. The common skin diseases afflicting people and which are also treated with traditional medicines include: *Tinea capitis*, *Tinea corporis*, scabies, acne, *Erythema multiforme*, leg ulcers, localized vitiligo, seborrhoic dermatitis and all types of eczema [3,6,7].

Despite the enormous advantages in the treatment of these diseases, traditional medicines are also implicated for various side-effects [8] both internal and external to human and animals. Recently, a number of publications reporting dermal toxicity potential of various plant preparations used traditionally for skin applications have emerged. Ernest, 2000 [9] reported the potentials of many plants to cause dermal toxicity. In his review, he registered plants such as St John's Wort, *Piper methysticum* (kava), *Aloe vera*, *Eucalyptus* sp, *Cinnamomum camphora* (camphor), *Lawsonia inermis* (henna) and *Pausinystalia yohimbe* to have great potential of dermatological side effects. Other herbal treatments used for dermatological conditions such as Chinese oral herbal remedies for atopic eczema, have the potential to cause systemic adverse effects [9]. On the other hand

pure latex from the majority of plants has been found to exhibit severe skin irritations [4,5]. Many other organic plant extracts that have so far been studied revealed mild to moderate skin toxicity [4,5].

In Tanzania like many other countries, the use of traditional medicament for skin related problems in human and livestock are a common phenomenon. During the survey to determine the prevalence of skin disease in Tanzania, Satimia et al. [6] revealed the use of both traditional and modern medicine in the treatment of various skin disorders. Similarly Moshi et al. [10] reported the use of *Jatropha curcas*, *Ricinus communis*, *Zehrenia scabra* and *Tricalysia coriacea* medicinal plants in the treatment of various skin conditions in Kagera region in Tanzania.

Synadenium glaucescens is among of the Tanzanian traditional medicinal plants which is known for many traditional application in human and animals including healing wounds, boils, HIV, worms and application on the swollen lymph nodes of cattle suffering from east coast fever (ECF). The plant is endemic in the East African Region and occurs in Tanzania, Kenya, Democratic Republic of the Congo and Burundi. *Synadenium glaucescens* has been proven to have strong antiviral and antibacterial activities [11]. Other species within the same genus were earlier reported to exhibit molluscicidal and insecticidal activities indicating their potential as biopesticides [12].

Despite the recorded utilization of plants through the dermal route in human and animals, there is scanty information regarding dermal toxicity of Tanzanian medicinal plants. This is in comparison with the amount of information that exists on cytotoxicity of different medicinal plants using the brine shrimp lethality test [13-14].

The aim of this study was to evaluate the toxicity potentials for of *S. glaucescens* commonly used for treatment of dermatological conditions in humans and livestock.

2. MATERIALS AND METHODS

2.1 Plant Materials

Plant materials (leaves and root barks) of *Synadenium glaucescens* (S.G) Pax were harvested from Mufindi District in Tanzania during May and August 2012. The World Health Organization (WHO) guideline on Good Agricultural and Collection Practices (GACP) for medicinal plants was used [15]. Thus, roots were dried under room temperature while some minor modifications were considered for leaves in which drying was effected in place with half day shade and half day sun due to the fact that leaves for this plant contain latex. The dried plant materials were pulverized and then subjected to solvent extraction with different polarities sequentially in ascending order starting with hexane, dichloromethane, ethyl acetate, methanol and ultimately water. After filtration, the

extracts were dried *in vacuum* and in a freeze dryer to obtain different organic and water extracts, respectively (Table 1).

2.2 Management of Experimental Animals

Healthy male white New Zealand rabbits (1.4-2.3 kg), healthy female adult wistar albino rats (71-89 g) and healthy female young adult albino mice (15-27 g) were used in the dermal toxicity for acute dermal irritations, acute dermal toxicity and skin sensitization studies, respectively. All animals were obtained from Soikoine University of Agriculture (SUA), Department of Animal Sciences and Production (DASP) for rabbits and in the Faculty of Veterinary Medicine for rats and mice. Rabbits were caged individually while rats and mice were caged in groups; all animals were supplied with conventional laboratory diets and water at *ad libitum* [16,17]. All female animals were nulliparus and non-pregnant. Housing was maintained at 22°C±3°C temperature and 40-65% relative humidity with a 12 hours light-dark cycle. Prior to the tests, animals were acclimatized in a laboratory condition for at least a week [16,17].

2.3 Laboratory Procedures

2.3.1 Acute dermal irritation tests

Acute dermal irritation tests were performed using the Organization for Economic Cooperation and Development (OECD) guidelines [16].

Table 1. Extract type and codes

Codes	Plant part	Extract type
GR-297-66201B	S.g (Root)	Dichloromethane (DCM) extract of S.g root prepared by extracting plant with DCM, after the plant materials extracted by Hexane
GR-297-66201C	S.g (Root)	Ethyl acetate (EOAc) extract of S.g root prepared after sequential extraction with Hexane and DCM and plant residue extracted with EtOAc
GR-297-66201D	S.g (Root)	Methanol (MeoH) extract of S.g prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with water (H ₂ O)
GR-297-66201E	S.g (Root)	Water extract of S.g root after sequential extraction with the above solvents
GR-297-66202B	S.g (leaves)	DCM extract of S.g leaves prepared by extracting plant with DCM, after the plant materials having been extracted by Hexane
GR-297-66202C	S.g (leaves)	EtOAc extract of S.g leaves prepared after sequential extraction with Hexane DCM, EtOAc; and plant residue extracted with EtOAc
GR-297-66202D	S.G (leaves)	MeoH extract of S.g leaves prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with H ₂ O
GR-297-66202E	S.g (leaves)	Water extract of S.g leaves after sequentially extracted with above solvents
GR-297-6603A	S.g (Root)	Ethanol Extract; fresh ground root barks extracted with ethanol

Twenty four hours before the test, fur from the backs of all rabbits was clipped using electric clipper exposing approximately 6cm² of skin [16]. Half a gram of each sample moistened with water and in some cases with a drop of sunflower cooking oil was evenly and gently applied using a small spatula in a test site while distilled water and in some case mixed with a drop of sunflower cooking oil was applied on the control side in this case the right side. Both sides were then covered using gauze of cotton cloth followed by a plastic sheath and then supported in place by a non irritating adhesive tape. After 4 hours the coverings were taken out and the test substance washed using distilled water. In cases where the substances were hard to get out especially for organic extracts, sunflower cooking oil was first used to soften and then water applied for cleansing. The test sites were then examined at 1, 24, 48 and 72 hours for dermal reaction using Draize scoring criteria (Table 1).

The Primary Irritation Indexes (PII) of test substances were then calculated using the formula below

$$(PII) = \frac{\text{sum of erythema/oedema}}{\text{No of test sites x grading interval}}$$

And the long form of the formula is;

$$PII = \frac{\sum \text{erythema grades at 1, 24, 48 and 72 hr} + \sum \text{edema grade at 1, 24, 48 and 72 hr total}}{\text{number of observations (number of animal) x observation intervals (4) x 2 (erythma and oedema)}}$$

The extract was then classified according to Draize method of classification using the PII scoring as mildly irritant if (PII < 2), moderately irritant (2 ≤ PII ≤ 5), and severe irritant (PII > 5)

2.3.2 Acute dermal toxicity tests

2.3.2.1 Selection of dosage

In acute dermal toxicity studies, only leaf extracts were tested. These extract included, water, methanol, ethyl acetate and dichloromethane respectively denoted as GR-297-6602E, GR-297-6602D, GR-297-6602C and GR-297-6602B. Selection of dosage was guided by procedures stipulated in OECD draft guideline no 434. Sighting study was conducted to all extracts tested. The limit dose of 2000 mg/kg was selected for the main study based on the fact that the 1000mg/kg as a start dose in the sighting study could not show any sign of toxicity when considering animal weight changes for two weeks.

2.3.2.2 Animals and preparation

The test in the albino rats were performed according to the OECD draft guideline number 434 [17]. A total of ten animals (all females) were divided into two groups of five animals each for a treatment and a control groups. Approximately 24 hours before the study, fur was removed from the dorsal area of the trunk of the animals by clipping to obtain at least 10% of the body surface area while taking care to avoid abrading the skin. Depending on the type of extract; the test substances were moistened with either sunflower cooking oil or distilled water then applied uniformly over a shaved area using a small spatula. Liquid test substances were used undiluted. The test substances were held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. At the end of the exposure period, residual test substance was removed using water or sunflower oil or both. Cage side observation was made daily, but weight measurement was taken weekly for 15 days. Observation included evaluation of skin and fur, eyes, respiratory effects, salivation, diarrhea, urination, and central nervous system effects (tremors and convulsion, gait and posture, reactivity to handling or sensory stimuli and altered strength). By the 15th day rats were humanely sacrificed and organs were carefully taken out for gross and histopathological examinations.

2.3.2.3 Gross and histopathological examination

Organs (Kidney, liver and lungs) were processed for histopathological examination using a standard procedure [18]. The organ tissues were first examined grossly for any observable effects and then sections (5 μM) were fixed in 10% buffered formalin embedded in paraffin, stained with haematoxylin-eosin (HE) and examined under light microscope. Tissue samples were then evaluated for degree of deformation and necrosis. The histopathological pictures of tissues from the different animal groups were evaluated by a pathologist and pictures taken using a digital camera.

2.3.3 Dermal sensitization studies

2.3.3.1 Selection of appropriate solvent

The criteria for selecting the right solvents were based on solubility of the test substances [4]. The procedure involved dissolving the extract in different trial solvents under both room and

raised temperatures using water bath. The solvents dissolved the largest part of the extract were selected as dissolution solvent for both sighting and actual tests. Thus, a range of organic solvents were tested. The extracts tested for solubility included dichloromethane (DCM) and ethyl acetate for both root barks and leaves. DCM extracts exhibited good solubility in chloroform and acetone while ethyl acetate extracts were soluble in ethanol. Therefore, acetone and ethanol were selected respectively as solvents for DCM and ethyl acetate extracts

2.3.3.2 Range-finding irritation test

Using the selected solvent, range-finding irritation test was performed and appropriate concentrations selected [4,16]. Sixteen female mice were randomized into eight groups; the groups were then randomly placed into different concentrations, two animals per each concentration. 100 µl of the respective concentration of the test solution was applied to the backs and area was then immediately dried using an electric drier. Ten microlitres of the respective concentration of test solution was then applied to the dorsal and ventral regions of the left ear and dried immediately. The mice were returned to their cage and left undisturbed for overnight. On day 1, each mouse was anesthetized with diethyl ether and thicknesses of both ears were measured and results were recorded. On the same day immediately and on subsequent days (days 2 and 3) 100 µl of the test solvent were again applied at the backs and on day 4, the skin of all animals was inspected for dermal irritation and scored as described above. The mildly irritant concentration on the belly regions (8 mg/ml) was chosen to be the dose for induction of the actual test and the highest non-irritant concentration to the ear (10 mg/ml) was chosen to be the challenge dose of the actual sensitization test [19,20].

2.3.3.3 Actual tests

The Mouse ear swelling test (MEST) method (C) by Sailstad et al. [21] was used to evaluate the sensitization potentials of some extracts from *S. glaucescens*. Thus, female mice were anesthetized using ether and the back of each mouse was shaved using small hair clipper [21]. Test chemicals or vehicle was applied: 100 µl without Freund's Complete Adjuvant (FCA) for 2 consecutive days. Chemical challenge occurred on day 6.

2.4 Data Analysis

Analysis of data varied depending on the study. Data from irritation were presented as scores from observation based on the Draize scoring criteria (Table 2.) after which PII was then calculated. Data from acute dermal toxicity were analyzed using Statistical Analysis Systems (SAS) software version 9.3 and the results expressed as mean weight \pm standard error. The student *t*-test was used to perform statistical tests and if the statistical power (*p*) was less than 0.005, the change was considered significant and the drug was considered less toxic. In the sensitization study, only the ear diameter was taken before and after sensitization and challenge. The mean diameter were calculated using excel and then compared to control.

Table 2. Draize scoring criteria for erythema and oedema

End point measured	Grade
No erythema /oedema	0
Very slight erythema/ oedema (barely perceptible)	1
Well defined erythema/ oedema	2
Moderate to severe oedema	3
Severe erythema/ oedema (beef redness) to eschar formation preventing grading of erythema	4

Maximum value is 4

3. RESULTS

3.1 Dermal Irritation

The extracts tested for dermal irritations were ethyl acetate, dichloromathene and water preparations from roots barks and leaves. Additionally, methanol leaf extract was also tested for irritations. The highest primary irritation index (PII) recorded in all extracts was 2.00 from the ethyl acetate of leaves while the lowest was 0.03 from water extracts of leaves. Thus, all of them fall into mild ($PII < 2$) and moderate ($2 \leq PII \leq 5$) irritant categories. The individual irritation scores (Table 3a and b) shows that the effects were pronounced in the first days but faded away with time and on 8 day's time almost all signs had faded away (Table 3a and 3b). None of the extracts from *S. glaucescens* could therefore be classified as an irritant.

From the findings, ethyl acetate and dichloromethane extracts were relatively more irritating than water and methanol extracts.

Table 3a. Average Irritation scores for leaf extracts of *S. glaucescens* in rabbits

Time intervals (hrs)	Draize scores (sums of erythema & oedema)			
	Ethyl acetate	Dichloromethane	Methanol extract	Water extracts
1	3.4	3	1.2	0.2
24	3.2	2.2	0	0
48	3.2	3.2	0	0
72	3	3	0	0
8th day	0	0	0	0
16th	0	0	0	0
PII	2.0	1.43	0.15	0.025

Table 3b. Average Irritation scores for root extracts of *S. glaucescens* in rabbits

Time intervals in hours	Draize scores (sums of erythema & oedema)		
	Ethyl acetate	Dichloromethane	Water extracts
1	3.4	4.4	2
24	1.8	3	0.2
48	3	3.4	0
72	2.8	1.6	0
8th day	0	0	0
16th	0	0	0
PII	1.375	1.55	0.275

3.2 Dermal Sensitization

Non occluded sensitization was performed using chloroform and ethyl acetate crude extracts from root barks and leaves of *S. glaucescens*. Eight milligram per milliliter (8 mg/ml) was selected as induction dose (The mildly irritant concentration on the belly regions) and 10 mg/ml (highest non-irritant concentration to the ear) selected as a challenge dose of the actual sensitization test 1 [9,20]. Each test extract was validated using a negative control and the right ear which was the non challenged ear. The results indicate that no sensitization was observed in any of the extracts for both test and negative control after 24 and 48 hours of post challenge.

3.3 Acute Dermal Toxicity

3.3.1 Selection of dosage for acute dermal studies

A starting dose of 1000 mg/kg in the sighting study could not show any sign of toxicity when considering animal weight changes for two weeks (Fig. 1a). The few observable weight losses for some of the animals in the 2000 mg/kg dosage was taken as toxic signs from the plant extracts and therefore was the main criterion for its selection. The drop of weight was obvious in the first week but rose to normal in the second week (Fig. 1b).

3.3.2 Clinical observation and mortality

Few hours after application of extracts many animals from the test groups showed some signs of discomfort and restlessness manifested by movements around the cage and limited intake of food and water. However, the eating and drinking slowly resumed in the same day and after removal of occlusion and test drugs, in the next day all animals resumed to normal state. Despite the discomfort, no adverse clinical signs were evident from all the animals in the test and control groups (Table 3). Albeit no scoring was done, the observable irritations caused by extracts were ranked to be mild or moderate with DCM and ethyl acetate extracts ranging highest. None of the animals showed any signs of edema from any of the extracts and control groups. Observation indicated that none of animals was found neither in a moribund condition nor showing any severe pain and/or enduring signs of severe distress [17]. Further observation was conducted for appearances of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma [17] and none of these signs was observed to any single animal in all the tests and control groups (Table 3).

3.3.3 Body weight trends

The means of weights of individual animals in the test groups are shown in Table 3 while the trend in weight change in the two weeks is shown in

Fig. 2. The results show significant mean weight increase in week one (P = 0.0003) from the initial weight. However, the change in weight of in the second week was not significant.

3.3.4 Gross pathology

Gross pathological examination of organs revealed no difference that could be established between test and control groups thus, no evidence of organ toxicity was associated to the extracts.

3.3.5 Histopathological examination

Histopathological examinations of organs also revealed no lesion suspected from drug effects in any of the test and control groups. All organs (kidney, liver and lungs) were normal with none of them showing degenerative changes of parenchyma, only blood vessel congestion in both control and test groups which were not linked to any chemical effect was evident (Figs. 2 and 3)

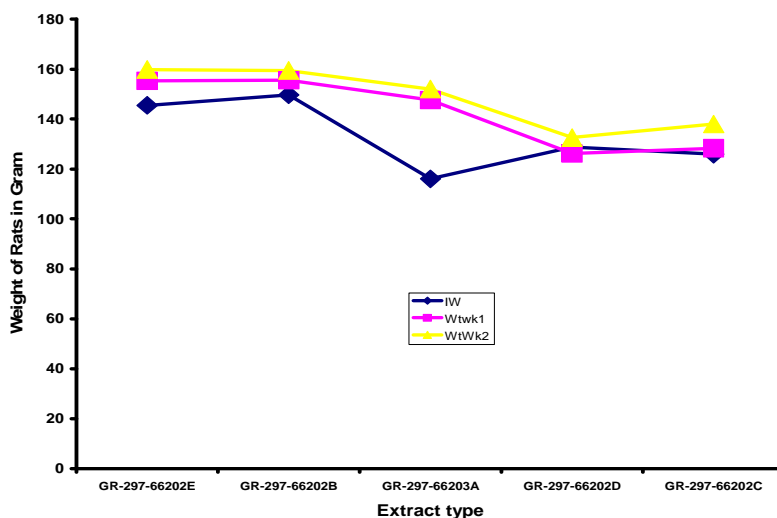


Fig. 1a. Weight change during sighting study for dermal toxicities for 1000mg/kg

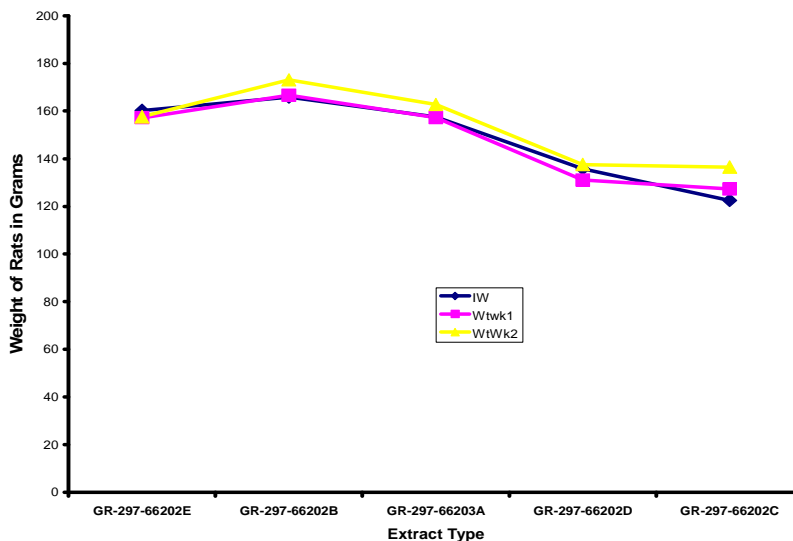


Fig. 1b. Weight changes during sighting study for dermal toxicities for 2000mg/kg

Table 4. Average Weight during the actual acute dermal toxicity tests

Week 1				
Extract code	Means ± std dev (gm)	P=0.05	Mean Cwtw1 (gm)	p>0.05
GR-297-6602B	99.42±11.64		24.40±3.64	
GR-297-6602B	106.78±10.24		23.98±2.51	
GR-297-6602B	108.38±6.86		28.86±4.06	
GR-297-6602B	120.62±10.90		23.96±8.98	
Control (Dist H ₂ O)	94.80±10.58		11.18±2.29	
Overall	106.00±0.11	***	22.48±0.34	***
Week 2			Cwtw2	
GR-297-6602B	111.22±11.46		11.80±4.54	
GR-297-6602C	117.04±12.48		10.26±2.67	
GR-297-6602D	119.38±10.54		11.00±4.98	
GR-297-6602E	133.54±13.71		12.92±3.34	
Control (Dist H ₂ O)	109.80±8.52		15.00±6.66	
Overall	118.20±0.26	NS	14.82±0.70	NS

Cwtw1 = change of weight one week after treatment from initial weight, Cwtw2 = change of weight one week from weight in week one

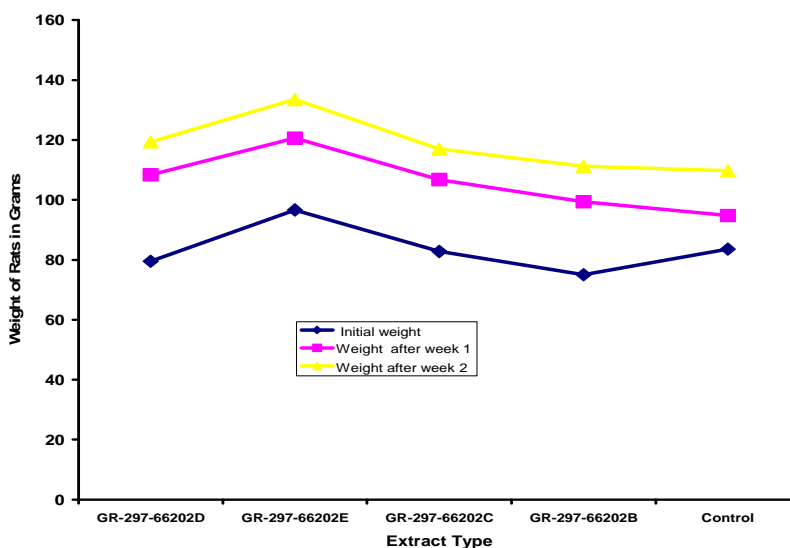


Fig. 2. Weight change during actual dermal toxicities tests

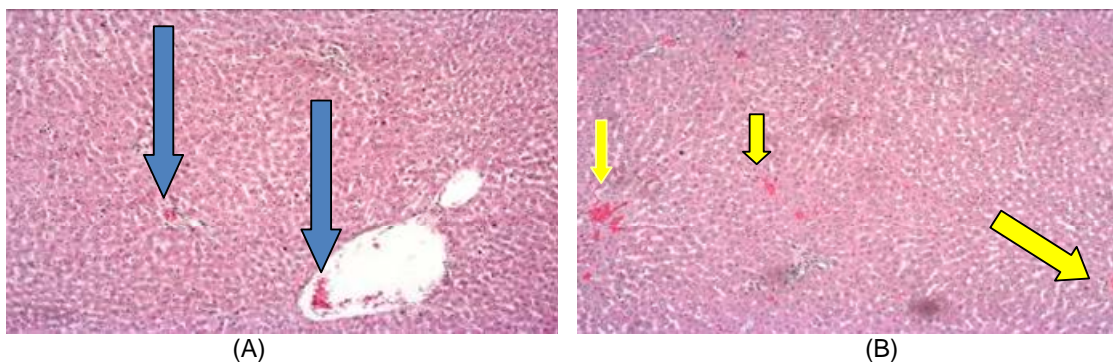


Fig. 3. (a) Photomicrograph of liver (a) from Methanol extracts (Blue arrows), (b) from distilled water ((yellow arrows) showing central vein and small blood vessel congestion H&E, X10 magnifications

4. DISCUSSION

4.1 Dermal Irritation

From this study, the calculated primary irritation indexes (PII) ranged from 0.05–3.2 for test substances and zero for control. Methanol extracts from leaves and water extracts from both leaves and roots exhibited PII of less than 2. According to Draize, [22] classification, these tree products were considered to have mild irritant effects. The other four extracts from dichloromethane and ethyl acetate extracts which are mid polar fractions from both plant parts were more irritant compared to polar fractions of water and methanol. These were concluded to have a moderate irritant effect since the PII was greater than 2 but less than 5. This could probably be due to fact that much of ingredients with irritant characteristics were contained in these portions of extracts. Elsewhere, similar findings have been obtained from other plant species of this family. Bigoniya et al. [5] reported a mild to moderate irritation in medium and polar fractions of *Euphorbia neriiifolia* but very severe irritations from fresh and dried latex [5]. The dermal irritation tests for fresh latex was not studied due to ethical grounds as high level of toxicity in most plants of the family Euphorbiaceae is found in the fresh latex. The irritation of medium polar and polar fraction is associated with the presence of phorbol type diterpenes esters while the less polar fractions are generally reported to contain triterpenes which make them non irritant [23]. However, According to Draize, [22] classifications all extracts in this study are considered not severe irritants because the PII of less than 5.

4.2 Dermal Sensitization

Unlike in the irritation study, dermal sensitization revealed no effect that can be related to the test extract. The control group and the test groups had similar ear diameter and even the right ears of all groups appeared the same. These results indicate that although the extracts from this plant exhibit mild to moderate irritations (section 5.1), generally they are not sensitizers. The methodology used in this evaluation was shorter in terms of induction and challenge interval and had not been extensively utilized in dermal sensitization tests. However, it demonstrated a similar effect in the earlier study by Sailstad et al. [5].

4.3 Acute Dermal Toxicity Study

Acute dermal toxicity results indicate that all animals in the tests and control groups did not exhibit any overt clinical signs of toxicities. The discomfort Signs experienced initially could probably be due to mild irritations and experiences of foreign materials (extract, occlusion cloth and adhesive tape) in the body. The loss in body weight which is an important maker of gross toxicity as stated by Banerjee et al. [24] did not occur and this further indicates that the extract did not cause acute toxicity. The significant mean weight increase in week one ($P = 0.0003$) from the initial weight further confirms that no effects were caused by the test chemicals. The non significant weight change in week two likely emerged from normal growth retardation that occurs with time. Drastic toxicity or interference with absorption of nutrient is normally reflected in body weight reduction [24]. No gross and histopathological lesion were observed.

5. CONCLUSION

Findings from this study have shown that, extracts from dried plant of *S. glaucescens* exhibit neither sensitization nor acute dermal toxicity effects except for mild to moderate irritancy. The findings therefore suggests that extracts from dried plant parts of *S. glaucescens* under the short term use of different extracts from dried leaves and root barks applied on skin of animals do not cause any adverse effects both externally and internally.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The research protocol was approved by the postgraduate studies committee of the Department of Veterinary Medicine and Public Health of the Sokoine University of Agriculture (SUA) in Tanzania.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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